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• LIVER CANCER •

Mitotic cell death in BEL-7402 cells induced by enediyne antibiotic lidamycin is associated with centrosome overduplication

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Abstract

AIM: Mitotic cell death has been focused on in tumor therapy. However, the precise mechanisms underlying it remain unclear. We have reported previously that enediyne antibiotic lidamycin induces mitotic cell death at low concentrations in human epithelial tumor cells. The aim of this study was to investigate the possible link between centrosome dynamics and lidamycin-induced mitotic cell death in human hepatoma BEL-7402 cells.

METHODS: Growth curve was established by MTT assay. Cell multinucleation was detected by staining with Hoechst 33342. Flow cytometry was used to analyze cell cycle. Aberrant centrosomes were detected by indirect immunofluorescence. Western blot and senescence-associated β-galactosidase (SA-β-gal) staining were used to analyze protein expression and senescence-like phenotype, respectively.

RESULTS: Exposure of BEL-7402 cells to a low concentration of lidamycin resulted in an increase in cells containing multiple centrosomes in association with the appearance of mitotic cell death and activation of SA-β-gal in some cells, accompanied by the changes of protein expression for the regulation of proliferation and apoptosis. The mitochondrial signaling pathway, one of the major apoptotic pathways, was not activated during mitotic cell death. The aberrant centrosomes contributed to the multipolar mitotic spindles formation, which might lead to an unbalanced division of chromosomes and mitotic cell death characterized by the manifestation of multi- or micronucleated giant cells. Cell cycle analysis revealed that the lidamycin treatment provoked the retardation at G2/M phase, which might be involved in the centrosome overduplication.

CONCLUSION: Mitotic cell death and senescence can be induced by treatment of BEL-7402 cells with a low concentration of lidamycin. Centrosome dysregulation may play a critical role in mitotic failure and ultimate cell death following exposure to intermediate dose of lidamycin.

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INTRODUCTION

Liver cancer is one of the most malignant tumors in the world[1,2]. Surgical resection is considered the most effective but not the most popular method for the treatment of hepatocellular carcinoma (HCC). Chemotherapy is indicated for a large member of HCC patients. Mitotic cell death is a cell death form different from apoptosis, on which has been focused in tumor therapy. It is also known as mitotic catastrophe or delayed reproductive death, and can be activated by radiation or antitumor agents at low doses or concentrations[3-5]. Mitotic cell death is frequently characterized by enlargement of cell volume, appearance of multi- or micronucleation, and arrest in G2/M phase of cell cycle. Finally, these cells underwent death. Thus far, little is known about the mechanism responsible for mitotic cell death. Some researchers considered that defects in mitotic machinery, such as multiple rounds of DNA synthesis without an intervening cytokinesis, and chromosome missegregation, might play a key role in the process of lethal nuclear fragmentation[6]. Previous reports have suggested that the absence or delay of the G1/S checkpoint and the subsequent absence of interphase apoptosis coupled to this checkpoint contribute to mitotic cell death[7,8].

The centrosome, representing the major microtubule organizing centre in eukaryotic cells, contains a pair of centrioles surrounded by pericentriolar material. The centrosome duplicates once during each cell cycle. To complete the normal cell cycle, the centrosome duplication cycle and the centrosome quantity must be precisely regulated to couple the other events of cell cycle[9]. If centrosome replication deviates from cycles of DNA synthesis and mitotic division, an unsuccessful mitosis will come out with the features associated with the formation of aberrant centrosomes and multiple mitotic spindles, and unbalanced chromosome segregation[10].

Enediyne antibiotics have been focused on their potent antitumor activity due to their unique ability to damage the DNA of tumor cells by inducing single strand (SSB) and/or double strand (DSB) breaks through free radical attacks on the deoxyribose moieties in DNA[11]. Lidamycin (also designated as C1027) is a member of the enediyne antibiotic family, which was isolated from a *Streptomyces globisporus* C1027 strain in China[12,13]. Lidamycin consists of a chromophore and an apoprotein, and the former has the ability to attack DNA, whereas the latter plays the role as a protecting protein[14]. The biological effects induced by lidamycin and ionizing radiation are similar[11]. Previous reports have shown that lidamycin is highly cytotoxic toward tumor cells[14-16]. As an attempt to investigate the mechanisms of lidamycin-induced mitotic cell death in human hepatoma BEL-7402 cells, we treated cells with lidamycin at low concentrations, and discovered centrosome overduplication, multipolar mitotic spindle formation, multinucleation, delayed reproductive death and changed patterns of protein expression associated with the regulation of proliferation and apoptosis.

These results indicate that mitotic cell death in BEL-7402 cells induced by lidamycin is associated with centrosome overduplication independently of mitochondria pathway.

MATERIALS AND METHODS

Chemical

Lidamycin was generously provided by Professor Lian-Fang Jin from our institute, and stored at -20 °C as a 100 μmol/L stock solution in 9 g/L NaCl solution.

Cell culture

Human hepatoma BEL-7402 cells (obtained from the Key Laboratory of Cell Proliferation and Regulation Biology of the Ministry of Education, Beijing Normal University) were cultured in DMEM (Gibcol BRL) supplemented with 100 mL/L fetal bovine serum (HyClone), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in the presence of 50 mL/L CO_2.

Growth curve assay

Growth curves establishments were performed at a 5-d interval as previously described[17] with some modifications. Totally 2.0×10^3 cells were seeded into 96-well plates and then treated with lidamycin for 2 h. A 12 μL MTT (5 mg/mL) was added to each well before assay and incubated for an additional 4 h at 37 °C, followed by treatment with 100 μL of 0.01 mol/L HCl-100 g/L SDS overnight. The value at each time point was read on a Microplate Reader (Model 550, Bio-Rad) at λ_{570nm}.

Cell multinucleation shown by staining with Hoechst 33342

Subconfluent cells were continuously incubated for 72 h after exposure to lidamycin for 2 h, and then were stained by the DNA-specific fluorescent dye Hoechst 33342 (2 μg/mL) (Sigma) for 15 min at 37 °C. Next, cells were washed once, kept in PBS, and observed using a fluorescence microscope (BH2 system, Olympus) equipped with a λ_{455nm} filter.

Flow cytometry

Cells were exposed to 0.5 nmol/L lidamycin for 2 h and then incubated in fresh, drug-free medium. Following a 3-d incubation, cells including the floating and the attached were harvested and washed with cold PBS twice. Cell suspensions were fixed in 700 mL/L ethanol at 4 °C overnight. Next, the fixed cells were washed twice in PBS and incubated with 50 μg/mL RNase (Sigma) for 30 min at 37 °C. Samples were then stained with 50 μg/mL propidium iodide (Sigma) in the dark at 4 °C for 30 min, and analyzed on a fluorescence-activated cell sorter (EPICS XL, Coulter).

Indirect immunofluorescence

Cells were grown on coverslips. After 3 d following 2-h lidamycin treatment, the cells were washed in PHEM buffer (60 mmol/L PIPES, 25 mmol/L HEPES, 10 mmol/L EGTA and 2 mmol/L $MgCl_2$) twice briefly, and incubated with a permeabilization buffer (5 mL/L Triton X-100 in PHEM buffer) for 90 s. Then, the cells were fixed in 37 g/L paraform in PHEM buffer for 15 min at room temperature. After washed in PBS 3 times, the cells were incubated with a blocking solution (50 g/L defatted dry milk and 0.5 mL/L Tween-20 in PBS) for 30 min and used for indirect immunofluorescence. The primary antibodies included anti-α-tubulin monoclonal antibody (Zymed) and anti-centrin polyclonal antibody[18] (kindly provided by Professor Da-Cheng He, the Key Laboratory of Cell Proliferation and Regulation Biology of the Ministry of Education, Beijing Normal University). Rhodamine-labeled goat anti-mouse antibody (Zymed) and Fluoresceinisothiocyanate-labeled goat anti-rabbit antibody (Zymed) were used as second antibodies. The microscope slides were mounted with glycerol mounting medium (900 mL/L glycerol and 100 mL/L PBS) and observed under a laser-scanning microscope (IX-70 system, Olympus). The cell with three or more centrosomes was considered aberrant.

Senescence-associated β-galactosidase (SA-β-gal) staining

Cells were treated with 0.1 nmol/L or 0.5 nmol/L lidamycin for 2 h and continuously maintained for 72 h. The attached cells were fixed in 5 mL/L glutaraldehyde and stained for SA-β-gal activity using X-gal at pH 6.0 as previously described[19].

Western blot analysis

Cells incubated with lidamycin at 37 °C for 2 h and then allowed to recover for 72 h at 37 °C were harvested and washed in PBS. The cells were lysed on ice in lysis buffer (100 mmol/L Tris, pH 6.8, 25 g/L SDS, 100 mL/L β-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, and 100 mL/L glycerol) for 10 min, followed by ultrasonication. The cell lysates were cleared by centrifugation, and the protein concentration was estimated using the Bradford method with bovine serum albumin as a standard. Western blot analysis was performed as a protocol described previously[20]. In brief, equal amounts of protein were electrophoresed on SDS-polyacrylamide gel (Fluka) and transferred onto a nitrocellulose membrane (Hybond-P, Amersham Pharmacia) for blotting with primary antibodies including anti-Bax (N-20, Santa Cruz), anti-Smac (a kind gift from Dr. Xiao-Dong Wang, University of Texas Southwestern Medical Center, Dallas, USA), anti-cyclin B1 (GNS-1, Santa Cruz), anti-p16 (16P04, NeoMarkers), anti-Rb (C-15, Santa Cruz), anti-p53 (DO-1, Santa Cruz), anti-p21 (F-5, Santa Cruz), and anti-actin (I-19, Santa Cruz) antibodies. Secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia). Enhanced chemiluminescence (ECL Western Blot Kit, Amersham Pharmacia) was used according to the manufacturer's instructions.

RESULTS

Growth inhibition induced by lidamycin in BEL-7402 cells

Inhibition of cell growth and proliferation was measured by the MTT test. Exposing BEL-7402 cells to 0.1 and 0.5 nmol/L lidamycin resulted in a dose-dependent inhibition of cell growth (Figure 1).

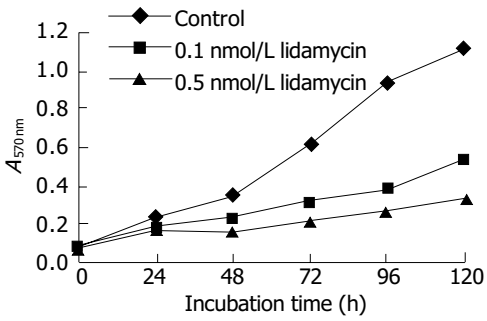

Figure 1 Effects of lidamycin treatment on BEL-7402 cells growth. Cells were seeded in 96-well plates and treated with 0.1 nmol/L or 0.5 nmol/L lidamycin for 2 h. Growth curves were established indirectly by detecting reactions with MTT. The value of each time point was derived from three samples.

Atypical chromatin condensation and multinucleation induced by lidamycin in BEL-7402 cells

With 0.1 nmol/L lidamycin for 2 h, followed by a 72-h incubation in drug-free medium, the treated BEL-7402 cells displayed a unique and atypical chromatin condensation characterized by appearance of small "dots" representing segregated condensed chromatin without apoptotic bodies (Figure 2B). Moreover, we did not observe detachment of these cells from the monolayer during the process of chromatin condensation. After 72-h incubation, multinucleation (three or more nuclei), one of the

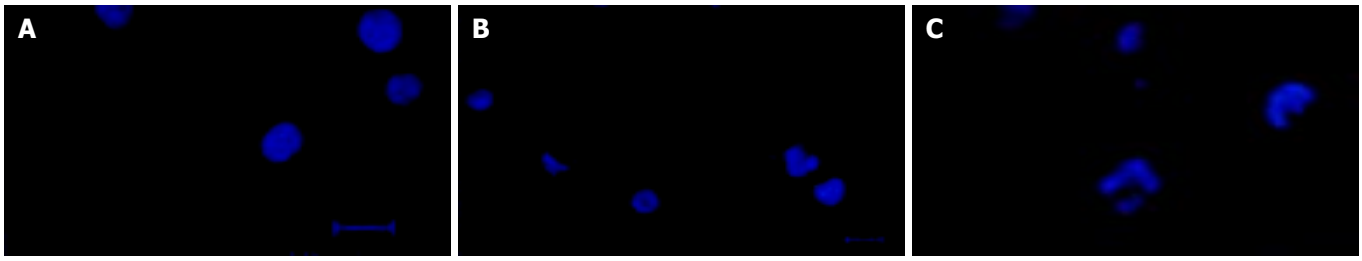

Figure 2 Induction by lidamycin of atypical chromatin condensation and multinucleation in BEL-7402 cells, determined by staining with the fluorescent dye Hoechst 33342 at 72 h after exposure to 0.1 nmol/L and 0.5 nmol/L lidamycin for 2 h. A: Untreated BEL-7402 cells; B: 0.1 nmol/L lidamycin treated BEL-7402 cells; C: 0.5 nmol/L lidamycin treated BEL-7402 cells.

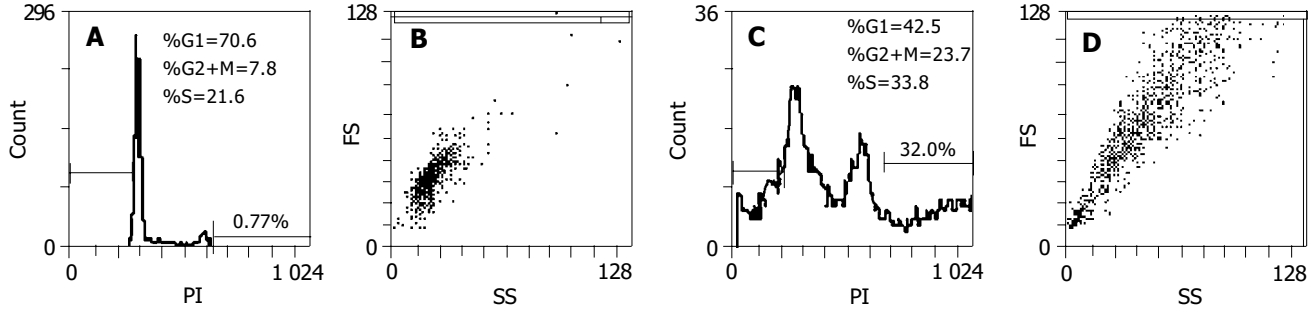

Figure 3 Effects of lidamycin on cell cycle. BEL-7402 cells were treated with 0.5 nmol/L lidamycin for 2 h, then washed and fed with fresh medium. After 72 h of incubation, the cells were stained with propidium iodide and sorted by flow cytometry. A,B: Untreated BEL-7402 cells; C, D: 0.5 nmol/L lidamycin treated BEL-7402 cells. The changes indicated in dot density maps were in accordance with those displayed in histograms.

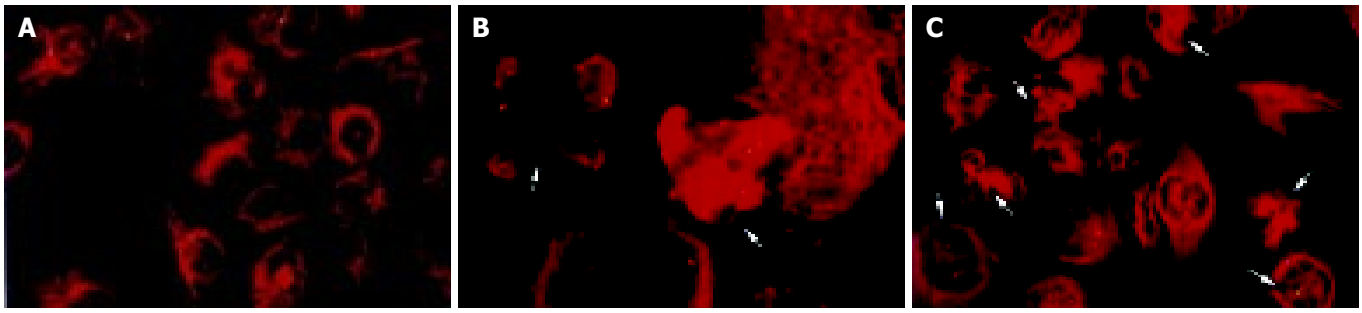

Figure 4 Immunofluorescence analysis of centrosomes and mitotic spindles in BEL-7402 cells at 72 h after treatments with 0.5 nmol/L lidamycin for 2 h. Cells grown on coverslips were fixed in paraform and doubly labeled with antibodies to α- tubulin (red) and centrin (green). Localization of centrosome on superimposed image showed yellow. A: Untreated BEL-7402 cells; B,C: 0.5 nmol/L lidamycin treated BEL-7402 cells. Arrows in B,C indicated the cells with multiple centrosomes, multipolar mitotic spindle or chromosome missegregation.

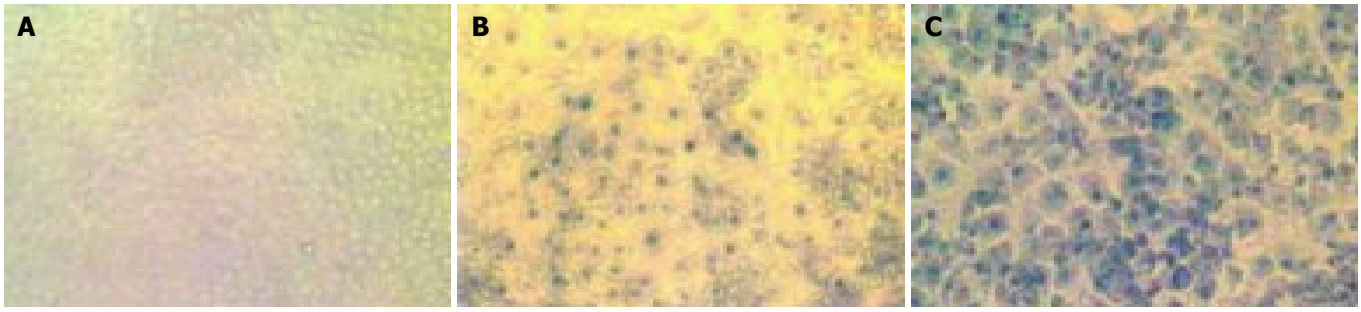

Figure 5 Phase contrast images of SA-β-gal stained BEL-7402 cells. At 72 h after incubation with lidamycin for 2 h, attached cells were fixed with 5 mL/L glutaraldehyde and stained for SA-β-gal activity for 16 h. A: Untreated BEL-7402 cells; B: 0.1 nmol/L lidamycin treated BEL-7402 cells; C: 0.5 nmol/L lidamycin treated BEL-7402 cells. The photographs were taken at 200-fold magnification (25 μm scale bars).

main features of mitotic cell death, occurred at 0.5 nmol/L lidamycin-treated BEL-7402 cells (Figure 2C).

Changes of cell cycle progression and DNA polyploidy induced by lidamycin in BEL-7402 cells

The biochemical and cytological changes of multinucleated giant cells remain poorly understood. To further characterize the etiology of mitotic cell death, we analyzed the cell cycle and DNA content of the lidamycin-treated BEL-7402 cells by flow cytometry. The cells were exposed to a low concentration of lidamycin for 2 h. At 72 h after treatment, ~23.7% of BEL-7402 cells arrested in G2/M phase, and the cells with >4N DNA

content were detected at 32.0% (Figure 3C).

Centrosome overduplication, multipolar spindle formation and unbalanced division induced by lidamycin in BEL-7402 cells

Immunofluorescence microscopy revealed that the abnormalities of multiple centrosomes and multipolar mitotic spindles markedly increased in BEL-7402 cells induced by lidamycin at a low concentration. Co-staining with antibodies to centrin and α-tubulin indicated that the increased centrosomes were localized at each pole of the multiple spindles, and the cells not deriving from equal division appeared (Figure 4B, C). However, to untreated cells, these defects rarely displayed (Figure 4A).

Induction by lidamycin of senescence-like phenotype in BEL-7402 cells

The induction of SA-β-gal activity and mitotic cell death were thought to be independent events[21]. To investigate the effects of mitotic cell death on senescence-like phenotype of the lidamycin-treated cells, we observed SA-β-gal expression, a senescence marker, at 72 h after lidamycin treatment. The treated cells showed phenotypic changes that resembled features of normal senescence, including enlarged and flattened morphology, increased granularity, vacuolization, and enhanced SA-β-gal-positive cells (Figure 5). Moreover, the induction of senescence-like phenotype in BEL-7402 cells was increased dose-dependently.

Alterations of cell cycle related proteins in lidamycin-treated BEL-7402 cells

To understand some molecular changes that led to mitotic cell death, cell cycle proteins were analysed by Western blot analysis in lidamycin-treated cells. In BEL-7402 cells, lidamycin induced an increase in the levels of cyclin B1, p16 and pRb, meanwhile a decrease in expression of p53 and p21 (Figure 6).

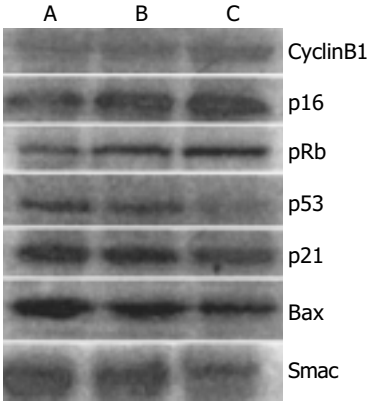

Figure 6 Western blot analysis of BEL-7402 cells. Cells were harvested at 72 h after 2-h lidamycin treatment, and total proteins (30 μg/lane) were resolved by 8 to 15% SDS-PAGE and transferred onto nitrocellulose, and Western blot was performed using the indicated antibodies. Lane A, untreated BEL-7402 cells; lane B, 0.1 nmol/L lidamycin treated BEL-7402 cells; lane C, 0.5 nmol/L lidamycin treated BEL-7402 cells.

Alterations of apoptosis related proteins in lidamycin-treated BEL-7402 cells

To confirm that mitotic cell death induced by lidamycin was distinguished from typical apoptosis, and detect the correlation between them, proteins associated with apoptosis were examined. Bax is a regulator of apoptosis[20]. Smac, an inhibitor of caspase suppressors, promotes cytochrome c-induced activation of caspases by sequestering the inhibitor of apoptosis protein family[22]. As is shown in Figure 6, the protein levels of Bax and Smac declined in lidamycin-treated BEL-7402 cells. We were unable to detect the proteolytically activated caspase-3 and caspase-9, and found no significant alterations of the blots representing caspase-3 precursor and caspase-9 precursor after exposure to low concentrations of lidamycin in BEL-7402 cells (data not shown).

DISCUSSION

HCC is one of the most common malignant neoplasms in the world[1]. The incidence of HCC in China exceeds 100 000 per year, and at least 110 000 HCC-related deaths occur every year. Lidamycin is a highly potent cytotoxic antitumor agent. We treated human hepatoma BEL-7402 cells with lidamycin at concentrations in the nanomolar range, and assessed the potential role of centrosome in lidamycin-induced mitotic cell death. The results indicated a series of abnormal events including centrosome overduplication, formation of multipolar mitotic spindles, multinucleation, and eventual mitotic catastrophe. In this study, we have described for the first time the association between centrosomes and enediyne antibiotics-induced cell death in human hepatoma cells.

Thus far, the modes of cell death induced by lidamycin can be divided into two classes, one is apoptosis, and the other is mitotic cell death[5,23]. It has been reported that lidamycin can act directly as an endonuclease without dependence on caspase activities and is considered as an apoptosis-mimetic agent, at high concentrations[24]. Cells exposed to low concentrations of lidamycin lost reproductive integrity due to inappropriate entry into mitosis, and apparently exhibited the morphological and biochemical changes associated with mitotic cell death: enlarged cell shape, multi- or micronucleation, accumulation of karyotypic abnormalities, and a G2/M arrest[5]. Apoptosis, mitotic cell death and irreversible cell cycle arrest may all contribute to cell death after lidamycin treatment. The exact mechanism of mitotic cell death is unclear, and only a few studies have attempted to elucidate the effects of lidamycin on cells at moderate concentrations[5,11]. In the present study, we used low-dose lidamycin to treat BEL-7402 cells, and mitotic cell death was observed predominantly after treatment. We demonstrate that lidamycin can induce multiple centrosomes which may be responsible for the assembly of multipolar mitotic spindles and the chromosomes missegregation. Most of the cells containing multiple nuclear fragments were temporarily viable but reproductively dead. However, in some cases, the cells undergoing mitotic death initiated endocycles, restituted mitosis and finally survival[8]. We plan to continue this study to confirm the link between lidamycin-induced mitotic cell death and centrosome overduplication by using centrosome inhibitors and vectors containing antisense mRNAs to centrosome related proteins.

Because centrosome duplication was closely associated with DNA replication, cytokinesis and cell cycle regulation[25,26], we analyzed the cell cycle progression of lidamycin-treated BEL-7402 cells to detect relationship between centrosome dysregulation and cell cycle distribution as well as to confirm the appearance of multinucleated cells, which is one of the main features of mitotic cell death. We found that lidamycin induced centrosome overduplication associated with induction of G2/M arrest. Previous reports showed that centrosome replication could dissociate from DNA synthesis cycle and mitotic division[27], and cell cycle block in G2/M phase might be related to abnormal centrosome accumulation[10], and endocycles starting from G2/M arrest could produce endopolyploid cells[7]. We suppose that lidamycin-induced DNA replication cycle retardation in BEL-7402 cells could be helpful to trigger centrosome overduplication.

Analysis of gene expression may provide further insights into the molecular mechanisms mediating mitotic cell death. Cyclin B1, a component of the mitosis-promoting factor, plays an important role in G2/M regulation by forming a complex with

p34cdk1 to phosphorylate various substrates necessary for mitosis. The cells with the morphological features of mitotic catastrophe frequently undergo up-regulation of cyclin B1 level[28,29], which is consistent with our results. Bax expression is a regulator of apoptosis. Ordinarily action of Bax facilitates apoptosis[30]. However, our present study demonstrated that the levels of Bax and Smac both decreased along with no proteolytic activation of caspase-3 and caspase-9, and no DNA ladder was obtained (data not shown) after lidamycin treatments, which suggested that the mitochondrial apoptosis pathway might not be activated. The undetectable typical apoptosis is not caused by Bcl-2 involvement in BEL-7402 cells exposed to low concentrations of lidamycin (data not shown) and some other genes might play an essential role in this response. p21 is a p53-regulated protein. p53 inhibition was shown to increase mitotic death[31]. We noticed decreased levels of p53 and p21 in BEL-7402 cells after lidamycin treatment. However, the expression of p16 and pRb proteins was upregulated in BEL-7402 cells, which might explain the increased intensity of staining for SA-β-gal in lidamycin-treated cells, since p16 is closely related to induction of senescence-like phenotype[32]. Based on data presented here, we propose that the G2/M arrest of BEL-7402 cells may not be mediated by a classically driven cell cycle checkpoint mechanism correlated with p53. From a therapeutic standpoint, centrosome dysregulation might provide a valuable anticancer target. Further study to identify signaling pathways to mitotic cell death in tumor cells and normal somatocytes would help to improve the efficacy of HCC therapy with a low systemic toxicity.

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